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The role of asymptomatic secondary hosts in the epidemiology of Verticillium wilt

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Abstract

In this one-year feasibility study, we sampled *Verticillium dahliae* from its primary host (potato) and rotational crops and weed populations in the same fields to demonstrate the need for understanding the role of these asymptomatic secondary hosts in the epidemiology of Verticillium wilt. *V. dahliae* was isolated from 16 weed species in 11 plant families in the western Negev region of Israel, but from only one weed species in Pennsylvania (USA). In contrast, *V. dahliae* was not recovered from wheat and barley grown in rotation with potato in Israel, but was isolated from oats grown in rotation with potato in Pennsylvania. Isolates collected in Israel all belong to vegetative compatibility group (VCG) 4B, whereas those from potato in Pennsylvania are in three different VCGs (2A, 4A and 4B). DNA for most of the isolates recovered in this study were sent for genotyping by sequencing; not surprisingly, all genotypes are consistent with their grouping into the three VCGs, as expected in this highly clonal species. No differences in fungal genotypes were found between potato and secondary hosts (rotational crops and weeds). We developed a genotyping method based on next-generation sequencing (using the Illumina MiSeq platform) and 57 single-nucleotide polymorphisms (SNPs) diagnostic for clonal lineages in *V. dahliae*. This method correctly assigns isolates to clonal lineages. Further optimization is needed to adapt this method for quantifying the frequencies of mixtures of lineages in plant material or soil. This method has promise as a research or diagnostic tool where the identity of *V. dahliae* clonal lineages is essential.

Contribution of collaboration

This collaboration between US and Israeli scientists expanded the scope of our understanding about the biology of *Verticillium dahliae*. Interestingly, the results obtained in the two locations were quite different. In Pennsylvania, using nearly identical methods, we found *V. dahliae* almost exclusively in rotational oat crops, but very few samples from weeds. By contrast, sampling in Israel found that *V. dahliae* was relatively common in a diverse array of weeds, but not in rotational wheat crops. In both cases, however, we found the same genotypes in primary hosts (potato) as found in secondary hosts (weeds and oats). Whether these differences are caused by climatic factors in the two regions (Pennsylvania versus the Western Negev), by differences in rotational or weed hosts, or by differences in weed management is not known.

Our collaborative team exploited the strengths of each participating PI/co-PI. In each country we combined experts in *V. dahliae* biology/pathology with experts in population biology. The complementation of the diverse expertise, however, was excellent for our discussions and ability to formulate questions about *V. dahliae*. For example, analyzing *V. dahliae* populations by vegetative compatibility groups and genotyping by sequencing were done in different research groups. We look forward to a continuation of this collaboration in future funding cycles.

Major Achievements for one-year feasibility study

Isolation of *Verticillium dahliae* from weeds and nonhosts. We attempted to isolate *V. dahliae* from asymptomatic secondary hosts, particularly cereal and other rotational grass crops, and weeds in fields currently or previously planted to potato. Monocots are traditionally considered "nonhosts" and are asymptomatic when infected with *V. dahliae*; currently, little information is known about dicot weeds as hosts of *V. dahliae*.

In Israel, an extensive collection was conducted in the western Negev and the Judean low hills (Shfelat Yehuda) in 2015 and 2016, focusing mainly on wheat and barley fields with histories of potato production, and on the common weeds in these fields. *V. dahliae* was isolated from 16 species of weeds, in 11 families (Table 1, in Appendix), including common weeds in the western Negev region, such as *Amaranthus blitoides*, *Heliotropium hirsutissimum*, *Solanum elaeagnifolium,* and *Sinapsis arvensis*. In addition we isolated *V. dahliae* from *Sorghum bicolor* which is considered a nonhost. Despite our best efforts, we could not isolate *V. dahliae* from either roots or stems of more than 200 wheat and barley plants. In total, we obtained 42 isolates of *V. dahliae* in this collection (Table 1), all of which belong to vegetative compatibility group 4B (VCG4B). Interestingly, we were also able to isolate *Verticillium tricorpus* from potato and durum wheat, marking the first report of this weak pathogen from these hosts in Israel.

In the USA, similar collections were conducted in 2015 in Schuylkill County, Pennsylvania, the main potato-producing area in the state. Sampling was done on five farms with known histories of Verticillium wilt of potato that were rotating with nonhosts. In addition to potato and weeds, we sampled the following rotational crops: timothy grass (*Phleum pratense*), oat, rye, soybean, sudangrass, corn, and wheat. Depending on the crop, between 60 and 190 plants were sampled and subjected to isolation procedures for *V. dahliae*. Seven species of common weeds were evaluated for the presence of *V. dahliae*: *Apocynum cannabinum*, *Abutilon theophrasti*, *Chenopodium album*, *Oenothera biennis*, *Oxalis stricta, Euphorbia cyparissia,* and *Galinsoga parviflora.*

Despite extensive efforts and the use of two different semi-selective media for *Verticillium*, our rate of successful isolation was very low. We successfully isolated *V. dahliae* from potato, oat,

soybean, and common evening primrose (*Oenothera biennis*). In total we obtained 128 isolates (Table 1). Isolates from potato and oat were typed to VCG and placed in VCGs 2A, 4B, and 4A. The rest of isolates are currently being typed to VCG. *Verticillium*-like colonies were also observed in samples of sudangrass (roots only) and rye (root and stems), but due to the presence of other faster-growing fungi, pure cultures could not be recovered. We speculate that the unusually cold and wet conditions experienced during summer 2015 allowed other fungi (some of them pathogens), like *Fusarium* and *Colletotrichum* spp., to colonize plants, interfering with the isolation of *V. dahliae*. To test this hypothesis, we saved these plant materials and will extract total DNA (plant and colonizing microorganisms) for the molecular detection of *V. dahliae.*

Genotyping by sequencing (GBS). DNA was isolated from all isolates recovered in this study for GBS. In addition, we included reference isolates from Israel and Pennsylvania previously shown to be in known VCGs. Just prior to submission of samples in May 2016, Cornell University stopped providing GBS because of a licensing dispute. This resulted in lengthy delays in finding another vendor who could provide GBS services to produce results compatible with previous data obtained through Cornell. Data were delivered in late November 2016. Analysis of these data are consistent with previous results of this highly clonal species: SNP genotypes group the isolates strictly according VCGs; no recombinant genotypes were observed (Figure 1, in Appendix).

SNP genotyping to quantify the frequencies of *V. dahliae* lineages. Using GBS results obtained prior to this project, we identified single-nucleotide polymorphisms (SNPs) diagnostic for each of nine clonal lineages of *V. dahliae*. Our goal was to identify 4-8 SNPs for each lineage for developing rapid methods *a*) to assign isolates to lineages, and *b*) to detect the presence and estimate the frequencies of lineages in mixed environmental samples, e.g., DNA isolated from soil or plant tissue. Using multiplex PCR and amplicon sequencing (AmpSeq) using the Illumina MiSeq platform, we successfully identified 57 SNPs, which in aggregate are capable of assigning isolates to lineages, thus achieving our first goal. Unfortunately, attempts to quantify mixtures of lineages to date have not succeeded. Although we can *detect* mixtures of SNPs using AmpSeq, we cannot yet *quantify* lineage frequencies. Therefore, we have only partially achieved the second goal. Further optimization of PCR parameters will be needed to test this method. For example, we need to optimize the amount of template DNA and number of cycles to use in PCR.

In addition, we will explore the use of multiplex digital PCR for quantification of lineage frequencies. These optimizations and additional methodologies, however, could not be completed in the timeframe of this one-year feasibility study.

Significance of main scientific achievements or innovations. In this one-year feasibility study, we assembled a comprehensive collection of *V. dahliae* isolates in order to understand the possible role of weeds and nonhost rotational crops in the population dynamics of *V. dahliae* in the field. The results indicate that weeds from many families are serving as asymptomatic hosts of *V. dahliae* in general, and in potato fields in the Negev area and Pennsylvania in particular. The analysis of GBS data answered the question whether *V. dahliae* populations are genetically differentiated between the primary host (potato) and rotational crops and weed populations in the same fields: they are not differentiated. We recovered relatively few isolates from the rotational crops wheat and barley in Israel. This result may imply that our main hypothesis of rotational crops as a secondary host needs to be modified and focused on weeds instead. However, additional sampling should be performed with additional wheat varieties. In contrast, we did succeed in isolating *V. dahliae* from oats grown in rotations with potato in Pennsylvania, confirming their role, along with weeds, as potential sources of inoculum for potato crops. In addition, we developed a robust SNP genotyping method for identifying *V. dahliae* isolates to clonal lineages, which will streamline the VCG and GBS methods used previously.

Changes made in from the original research plan

We did not make any major changes from our original plan. However, we did have a few notable setbacks that meant we could not fully achieve all of our goals.

First, as mentioned the report on achievements, we were delayed in getting data from genotyping by sequencing (GBS) because of patent disputes that prevented Cornell University from performing GBS. However, we finally obtained the data in November 2016 and have analyzed the data (shown in the Appendix).

Second, the amplicon sequencing method (AmpSeq) we developed for assigning *Verticillium dahliae* genotypes to clonal lineages has stricter limitations than we had anticipated. Our method very successfully genotypes purified isolates of *V. dahliae*, but cannot be used for genotyping DNA from soil or plants, and cannot be used to quantify mixtures of genotypes. These findings means that our feasibility study is only partially successful in developing methods that we need for studying the population genetics of *V. dahliae* in experimental plots.

Publications for Project US-4839-15R

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun

Appendix

Table 1: Isolation of *Verticillium dahliae* from weeds and non-hosts crops from fields in the Western Negev region (Israel) and Pennsylvania (USA). All *V. dahliae* isolates from Israel were determined to be in vegetative compatibility group (VCG) 4B, whereas isolates from USA were in VCGs 2A, 4A, and 4B. DNA from these isolates have been sent for genotyping by sequencing (results not yet available).

Country	Type of host	Family	Host	Number of Isolates
Israel	Weed	Amaranthaceae	*Amaranthus albus*	6
		Asteraceae	*Calendula arvensis*	1
			Helianthus annuus	2
			Calendula arvensis	1
			Conyza bonariensis	1
		Boraginaceae	*Heliotropium hirsutissimum*	11
		Brassicaceae	*Sinapis arvensis*	3
			Diplotaxis erucoides	4
			Sinapis alba	2
			Rapistrum rugosum	1
			Sinapis sp.	1
			Erucaria sp.	1
		Convolvulaceae	*Convolvulus stachydifolius*	1
		Malvaceae	*Malva parviflora*	3

			Malva sp.	1
		Solanaceae	*Solanum nigrum*	1
			Solanum elaeagnifolium	1
	Asymptomatic crop	Poaceae	*Triticum durum*	1
			Sorghum bicolor	1
USA	Weed	Onagraceae	*Oenothera biennis*	1
	Asymptomatic crop	Poaceae	*Avena sativa*	28
		Fabaceae	*Glycine max*	12
	Symptomatic crop	Solanaceae	*Solanum tuberosum*	86

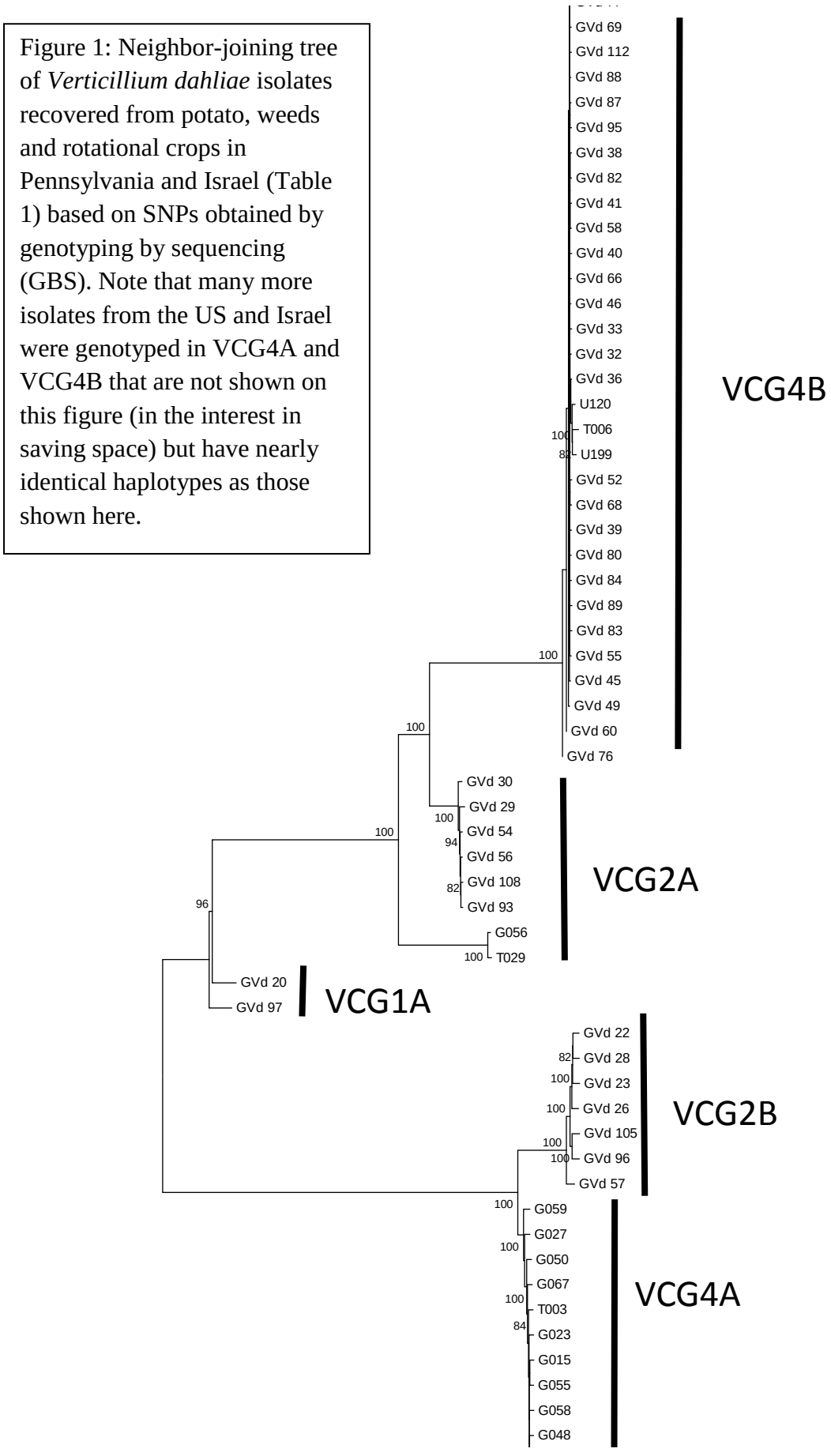

Figure 1: Neighbor-joining tree of *Verticillium dahliae* isolates recovered from potato, weeds and rotational crops in Pennsylvania and Israel (Table 1) based on SNPs obtained by genotyping by sequencing (GBS). Note that many more isolates from the US and Israel were genotyped in VCG4A and VCG4B that are not shown on this figure (in the interest in saving space) but have nearly identical haplotypes as those shown here.